Regulation of the fibronectin EDA exon alternative splicing. Cooperative role of the exonic enhancer element and the 5′ splicing site

A.F. Muro, A. Iaconcig, F.E. Baralle*

International Centre for Genetic Engineering and Biotechnology (ICGEB), Area Science Park, Padriciano 99, 34012 Trieste, Italy

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Abstract Alternatively spliced exons generally contain weak splicing sites, and exonic and/or intronic regulatory elements recognised by *trans*-acting auxiliary splicing factors. The EDA exon of the fibronectin gene is a typical example of an exon bearing a purine-rich exon splicing enhancer (ESE) element recognised by members of the SR phosphoprotein family. The regulatory region that governs splicing in the human EDA exon also contains an exon splicing silencer (ESS) element. We have cloned the mouse EDA genomic region, and we show that the ESE and the ESS elements, although they have base differences, can be replaced by the human elements without significant change in the exon inclusion/exclusion ratio. This fact suggests a common splicing regulatory mechanism across species. We demonstrate in vivo the functional activity of the mouse ESE element in splicing. We also show that the *trans*-acting factors recognising this element cooperate with the 5′ splicing site of the EDA exon to facilitate proper exon recognition. Indeed, a strong 5′ splicing site overrides the ESE function in exon recognition. However, the presence of a strong 3′ splicing site is not sufficient to compensate for the absence of the splicing enhancer. Our data provide in vivo evidence of the interplay between the exonic splicing regulatory elements and the splicing sites, leading finally to subtle regulation of alternative splicing.

Key words: Alternative splicing; Fibronectin EDA exon

1. Introduction

Pre-mRNA alternative splicing provides an additional source of genetic and biochemical flexibility for the cell [1]. The alternative splicing process can be regulated in a temporal, tissue-specific or developmental manner. The accurate excision of an exon from the pre-mRNA requires not only base pairing interactions between the 5′ splicing site and the 5′ terminus of U1 snRNA, and between the branch site and an internal region of U2 snRNA, but also a combination of *trans*-acting factors which are essential for the efficacy and precision of splicing.

Recently it was seen that exon sequences influence splicing site selection. A variety of exon splicing enhancer (ESE) sequences, stimulating the removal of introns with weak splicing signals, have been identified in constitutive and alternative splicing processes [2–6]. Many of these sequences contain a $(GAR)_n$ motif were R is a purine [7]. Different laboratories have shown that these enhancer elements are able to bind members of a family of serine/arginine-rich splicing factors named SR proteins [8–11]. The SR protein:pre-mRNA binding seems to be a very early step in spliceosome assembly [12]. Recent in vitro studies indicated that SR proteins promote the first step of spliceosome assembly assisting the association of the 5′ splicing site with the U1 snRNP [10,13,14] and the 3′ splicing site with the U2AF splicing factor [10].

The fibronectin (FN) gene represents an excellent system to study the molecular basis for alternative splicing [15–17]. Alternative RNA processing of human FN pre-mRNA occurs in three regions of the primary transcript called EDA, EDB and IIICS. EDA and EDB both encode a type III structural repeat and exhibit exon skipping while IIICS undergoes a complex splicing pattern that in humans generates five variants. As a consequence there are 20 different FN messengers, expression of which is regulated in a tissue-specific and developmental fashion as well as in response to physiological and pathological conditions.

EDA alternative splicing can be accurately reproduced in HeLa cells by transient expression of a three-exon hybrid FN-α-globin minigene [18]. Using this approach Mardon et al. [19] obtained the first evidence of the involvement of exonic sequences, not related to the splicing sites, in the mechanism of exon recognition. Deletion or replacement of 81 nucleotides located internally in the EDA exon abolished alternative splicing and resulted in the omission of the EDA exon from the mature FN mRNA. Previously, we and others have characterised the exonic sequences involved in the processing of the human EDA exon [2,8], describing that two distinct elements regulate the inclusion of the EDA exon with opposite effects. A short polypurine sequence (GAAGAAGA) acts as a splicing enhancer element (ESE) [2,8], while a downstream sequence (CAAGG) negatively affects the inclusion of the exons, thus acting as an exonic splicing silencer (ESS) [2]. Furthermore, Lavigueur et al. [8] have demonstrated that members of the SR protein splicing regulatory family interact with the polypurinic enhancer element of the EDA exon. The bipartite arrangement of *cis*-regulatory sequences seems to be common to other systems as recently established by other researchers [20–23].

Recently the presence of two additional exonic splicing regulatory elements in the EDA exon was reported, a silencer associated with a conserved RNA secondary structure, and a non-purine-rich splicing enhancer. Both elements are located in the 5′ region of the EDA exon [24].

There is a wealth of information regarding the mechanism of human fibronectin alternative splicing. Nucleotide sequence comparison with other species suggests a conservation of such a mechanism, although no study has been carried out up to date to experimentally confirm this assumption. The case of the mouse FN pre-mRNA processing is particularly interesting because the benefits of studying FN alternative splicing could be twofold. Firstly it will provide an extended knowl-

*Corresponding author. Fax: (39) (40) 226555.
E-mail: baralle@icgeb.trieste.it

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edge of the splicing mechanism and secondly it will make it possible to carry out very specific germ line manipulations that may shed light on the biological function of the FN isoforms generated by the alternative splicing process.

In the present work we characterise in vivo the purine-rich exonic enhancer element in the EDA exon of the mouse fibronectin gene, and extend the study of this system to the cross-communication that occurs between the ESE, *trans*-acting factors, and 5′ and 3′ splicing sites.

2. Materials and methods

2.1. Plasmids construction

A 12 kb FN fragment from a c129 genomic DNA lambda DashII library (a gift from Glenn Friedrich) was subcloned into pBSKSII and a 3988 bp *Kpn*I-*Kpn*I fragment subcloned into pBSKSII and completely sequenced. All DNA manipulations were done as described [25]. The numbering cited in the text refers to the *Kpn*I-*Kpn*I 3988 bp sequence. GenBank accession number AF095690.

A *Kpn*I site was introduced at position 220 by replacing the 3′ end of the EDA previous exon up to the *Pst*I site (nt 286) with a double stranded synthetic oligonucleotide. A *Not*I site was introduced in the EDA +1 exon at nt 2856 by PCR-directed mutagenesis. The complete 2656 bp genomic region comprising 77, 104, 1035 and 1170 of the EDA flanking exons and introns respectively, cloned at the *Kpn*I and *Not*I sites of the pBSKSII vector, was excised, made blunt and subcloned into the *Bst*EII site of pSVα1 [18]. This clone, containing the entire genomic region from the EDA −1 exon to the EDA +1 exon inserted in the third exon of the α-globin gene, was called pFN-EDAwt.

To produce the pFN-ΔEDA plasmid, the pFN-EDAwt was cut with *Nde*I and re-ligated, resulting in the excision of the 715 bp fragment containing the EDA exon. The *Nde*I 715 bp fragment was subcloned into the *Nde*I site of pET16, then *Xba*I-*Eco*RI cut and subcloned into pUC19 (clone 29) and PCR-mutagenised to obtain all the mutants. To obtain the ΔA mutation (GAAGAC deletion of the ESE, nt 1487–1492), a synthetic oligonucleotide containing the ESE deletion was introduced at the *Bsu*36I sites of clone 29. The resulting 709 bp *Nde*I-*Nde*I fragment was introduced in the *Nde*I site of the pFN-ΔEDA to generate the pFN-EDA-ΔA plasmid. The pFN-EDA-Δ76 and pFN-EDA-Δ88 deletion constructs were made by cutting clone 29 with *Bsu*36I, and partially digesting the ends with S1 nuclease, followed by ligation with T4DNA ligase. As described before, the *Nde*I-*Nde*I inserts were introduced in the pFN-ΔEDA plasmid.

The GAAGACGA (Am element) and CAGGG (Bm element) sequences of clone 29 were converted to GAAGAAGA (Ah) and CAAGG (Bh) respectively by PCR-directed mutagenesis. The 715 bp *Nde*I-*Nde*I resulting fragments were inserted into the pFN-ΔEDA plasmid to generate the pFN-EDA AmBh, pFN-EDA AhBm and pFN-EDA AhBh plasmids.

The 5′ splicing site mutant was created by converting the wild type CAG:GTATTG sequence to the 5′ splicing site consensus sequence CAG:GTAAGT by PCR-directed mutagenesis. The wild type 3′ splicing site of the EDA exon was replaced by the 3′ splicing site of the second exon of the human apolipoprotein (Apo) A1 gene {CCCTCC-TCCTCCTTCTTCTTCTTCAG:(A/G)}.

For double and triple mutant constructs, *Xho*I was used to interchange the 5′ and 3′ regions containing single or double mutations. The double 5′ splicing site mutant plus the ΔA deletion was made by PCR-directed mutagenesis as described above.

2.2. Cell transfections and RNA analysis

5×10^5 NIH3T3, Hep3B, HepG2, HeLa, N-Muli mouse liver or LMM3 mouse mammary [26] cells grown in 6 cm diameter plates were transfected with 5 μg of each construct by the calcium phosphate precipitate method or with lipofectin (Boehringer) as described [2]. The cells were harvested 40 h after the transfection and total RNA was prepared with RNAzol following the manufacturer's instructions. The cDNA was made using MMTV-RT and oligo-dT primer from 1 μg of total RNA. PCR reactions were done in linear conditions as previously described [2,27–29]. Briefly, the primers αG-FN dir (5′-C-ACTGCCTGCTGGTGACGTAC-3′) and αG-FN rev (5′-TGGGC-GGCCAGGGTCACGGC-3′) were used for the PCR reactions (60°C of annealing for 45 s, 72°C elongation for 45 s and 92°C denaturation for 45 s, for 30 PCR cycles). Both primers are specific for the mRNA transcribed from the transfected constructs and can discriminate the mRNA transcribed by host globin and fibronectin genes. The last 3′ bases match the residual linker sequences from globin and fibronectin exons. The PCR products were visualised in an ethidium bromide 1.6% agarose gel, photographed and subjected to densitometric analysis. The cell transfection experiments and RNA analysis were performed several times and representative experiments are shown in the figures.

3. Results

The murine genomic EDA region was cloned from a genomic c129 DNA library and comparison between the murine and the human EDA exon showed a perfect matching of the 3′ and 5′ splicing sites (Fig. 1), and revealed single base differences in each of the previously reported exonic regulators [2]. The polypurine sequence present in humans was interrupted in the mouse by a pyrimidine and also the putative mouse silencer splicing element contained one base change when compared with the human one (Fig. 1).

The splicing enhancer and silencer elements were originally defined in the human EDA by specific deletions, transient transfection of an α-globin-FN hybrid minigene and subsequent analysis of the transcribed mRNA [2,18,19]. We devised a similar approach to study the splicing mechanism governing the mouse EDA exon. The entire murine genomic region was cloned at the *Bst*EII site of the third exon of the α-globin gene and the minigene constructs were transiently transfected into different cell types (NIH3T3, Hep3B, HepG2, HeLa, N-Muli mouse liver and LMM3 cells).

Alternative splicing was detected after transient transfection with the pSVED-mEDA wt construct reflecting the endogenous alternative splicing pattern characteristic of the different cell types, and the splicing products obtained with each construct were similar in the various cell lines. The results obtained with NIH3T3 cells are shown in Fig. 2.

Constitutive exon skipping was observed when a deletion of the mouse polypurine element was tested, as shown in Fig. 2 (pSVED-mEDA-ΔA construct, lane 2). This result indicates that this element strongly stimulates splicing of the EDA exon. No significant differences were seen when the mouse GAAGACGA splicing enhancer sequence (Am) was mutated to generate the longer GAAGAAGA polypurine element (Ah) present in the human EDA sequence, as is the case for the pSVED-mEDA-AhBm construct (Fig. 2, lane 6). This reveals that the mouse exonic enhancer element stimulates splicing of the EDA exon in a similar manner as the human counterpart regardless of its shorter polypurine tract. Moreover, wider deletions of 76 and 88 bases centred in the A element completely abolish exon recognition (see Fig. 2, lanes 3 and 4, pSVED-mEDA-Δ76 and pSVED-mEDA-Δ88 constructs), having the same splicing efficiency as the pSVED-mEDA-ΔA construct.

In the pSVED-mEDA AmBh construct the CAGGG sequence of the mouse B element (Bm) was mutated to resemble the human one (Bh, see Fig. 1). Its splicing pattern was identical to the wt one. A similar result was obtained with the pSVED-mEDA AhBh construct, in which both A and B elements sequences were mutated to the human ones (Fig. 2, lanes 5 and 7). These results indicate that both the human polypurinic enhancer and the silencer element sequences are

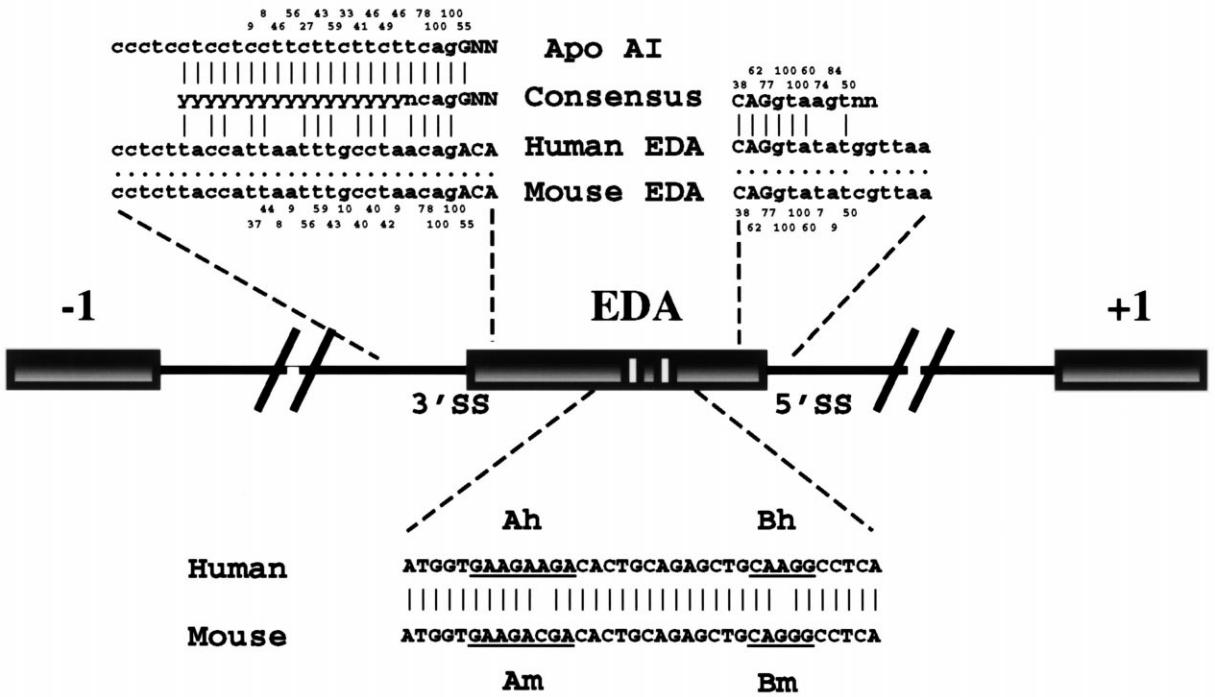

Fig. 1. Mouse vs Human comparison of the splicing elements that controls alternative splicing of the EDA exon. A schematic representation of the EDA region of mouse FN gene is shown. The sequence of the mouse and human 3′ and 5′ splicing sites is compared with the consensus sequence, and the exonic region sequence involved in EDA splicing regulation is indicated for the mouse and human exons. Enhancer (A element) and silencer (B element) sequences are underlined. Human and mouse elements are indicated as Ah, Bh, and Am, Bm respectively. Individual base frequencies are also indicated. FN exons and introns are shown as boxes and lines respectively.

functional in the mouse context, and may function via analogous mechanisms.

To understand the mechanism by which the ESE in vivo mediates enhancement of splicing we tested a series of constructs where the murine EDA wild type 3′ and 5′ splicing sites (SS) had been modified towards the consensus. The natural 5′ SS (CAG:GTATAT) was mutated to CAG:GTAAGT. This structural variant has improved base complementarity to U1 RNA. The 3′ SS of the FN-EDA exon was replaced by the 3′ SS of the constitutively spliced-in second exon of the Apo A1 gene (Fig. 1 and Section 2). This change should provide a stronger 3′ splicing site to the EDA exon.

The constructs with the consensus 3′ SS or the 5′ SS stimulated constitutive exon inclusion in transient transfection experiments (Fig. 3, lanes 3–5). The EDA exon was constitutively included in the construct where the natural FN 3′ or the 5′ splicing sites were replaced by stronger ones. As expected, this high splicing efficiency was also observed in a construct bearing both the 3′ and 5′ consensus splicing sites. Thus, increased U1 RNA complementarity and 3′ splicing site strength stimulate the rate of exon inclusion, resulting in both cases in uniform selection of the EDA exon.

Surprisingly when the ESE was deleted in the constructs shown above (see Fig. 3, lanes 8–12, pSVED-mEDA-ΔA-5′SScons, pSVED-mEDA-ΔA-3′+5′SScons and pSVED-mEDA-ΔA-3′SScons plasmids), constitutive exon inclusion was only obtained with the constructs bearing a strong 5′ splicing site. In effect, the pSVED-mEDA-ΔA-3′SScons constructs were not able to fully stimulate splicing in order to obtain constitutive inclusion (Fig. 3, lanes 11 and 12). It can be seen also that when the first base of the exon is a G instead of the A found in the wt sequence there is a stimulation in splicing, suggesting that the efficiency of spliceosome formation is affected in the construct containing the A as first base of the exon. In the pSVED-mEDA-ΔA-5′SScons construct,

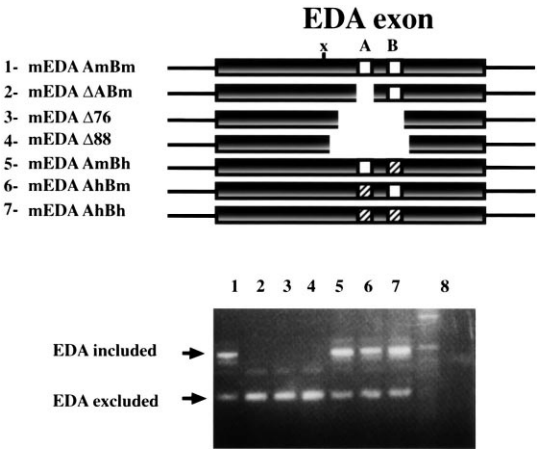

Fig. 2. Expression of the mouse EDA minigene variant in NIH3T3 cells. Schematic representation of the modifications introduced in the various minigene constructs. The thin lanes and shaded boxes represent intron and exon sequences respectively. The white or hatched boxes within the exons indicate mouse or human A and B elements respectively (upper panel). RT-PCR analysis of total RNA from cells expressing each of the indicated constructs in NIH3T3 cell line (lanes 1–7). Arrows indicate PCR products either containing or lacking the FN EDA exon in the messenger transcribed from the transfected minigene. Lane 8 corresponds to mock-transfected cells. A 1 kb ladder (BRL) was used as molecular weight marker (lower panel).

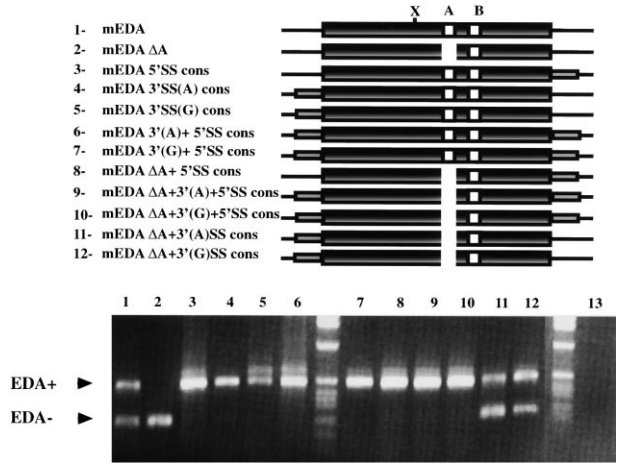

Fig. 3. Cooperation between splicing sites and the enhancer splicing element. Splicing site modified minigene constructs (upper panel) were transiently transfected into NIH3T3 cells and the produced RNA was analysed by RT-PCR (lower panel). Modified splicing sites are represented as light grey boxes at the splice junctions. Arrows indicate the splicing variants that contained EDA or skipped it. Lanes 1–12 show the analysis of the transfection of the different splicing-enhancer element mutant constructs. Mock-transfected cells are shown in lane 13. The A and B elements (A; B), and *Xho*I site are indicated (X). Lanes 3, 5, 6, 8, 9 and 10 show a minor larger extra band that corresponds to the 587 bp splicing product that utilises a 3′ cryptic site located 132 bases upstream of the natural EDA +1 exon 3′ splicing site. A 1 kb ladder (BRL) was used as molecular weight marker.

which has a deletion in the ESE and a strong 5′ splicing site, the EDA exon is spliced in with an exceptionally high efficiency (Fig. 3, lane 8), similar to the one obtained with the pSVED-mEDA-5′SScons or the any of the double splicing site modified constructs. Similar results were obtained by transfecting different cell types, regardless of the endogenous pattern of EDA alternative splicing (not shown). These results may indicate firstly that the SR proteins that recognise the ESE cooperate with the 5′ SS in the formation of the early splicing complex, and secondly that stimulation of spliceosome assembly occurs with the 3′ SS. Moreover, this cooperation seems to be a general mechanism in different cell types.

4. Discussion

The EDA exon sequence of the FN gene is highly conserved among different species, moreover the conserved splicing pattern may reflect a common evolution of the alternative splicing control mechanism. Despite these observations, comparison of the EDA sequences at the previously reported human exonic regulatory splicing elements showed single base changes in the core sequence of both the ESE and the ESS. Furthermore, comparison with other species also showed imperfect matching of the ESE sequences (see also Fig. 3 of Lavigueur et al. [8]). We have analysed if these minimal base changes were reflected as different regulatory splicing mechanisms in the mouse EDA exon. The introduction of the human sequence in the mouse A (ESE) and B (ESS) elements did not have significant consequences for splicing efficiency. Deletion of the mouse ESE has the same in vivo effect as the human counterpart. In fact, both in the human pSVED-Δ2e [2] and in the mouse pSVED-ΔA constructs (both bearing a deletion of the A element), the EDA exon was constitutively skipped.

It has been shown [8] that the purine-rich element of the human EDA exon in both in vitro splicing and gel shift assays favours the selection of the 3′ splicing site of the EDA exon by establishing U2 RNA and U2AF^{65} binding, and this effect is mediated by SR proteins specifically recognising this element. There are also other examples that show that SR proteins bound to exonic enhancers can promote an interaction of the U2 snRNA, U2AF^{65} and U2AF^{35}, promoting the recognition of the 3′ splicing site [30–32]. All these experiments were carried out in in vitro systems, utilising a two exons-one intron RNA transcript structure that does not resemble the three exons-two introns natural configuration of the EDA alternative splicing. The in vitro systems may overlook some of the fine mechanism that control alternative splicing. In fact, substrates having short truncated second exons function via direct intron definition, bypassing the exon definition model. Indeed, bypassing exon definition requires short introns and most in vitro experiments use internally deleted introns to maximise complex formation and activity [33]. In our case we utilised more physiological conditions, as in vivo assays, the complete genomic region containing the internal EDA exon plus its flanking exons, as well as the complete intronic intervening sequences. The results shown in Fig. 3 demonstrate in vivo that the ESE stimulates the recognition of the 5′ splicing site of the EDA exon. The presence of a strong 3′ splicing site is not enough to compensate for the absence of the ESE sequence. In contrast, a strong 5′ splicing site overcomes the ESE requisite. This may indicate that the *trans*-acting factors that recognise the ESE primarily cooperate with the 5′ splicing site, probably stimulating the binding of U1 RNA, as reported for the other examples [10,13,14], and secondly they could act as bridging factors across the entire exon to facilitate 3′ splicing site recognition. Furthermore, the cooperative role of the ESE and the 5′ splicing site seems to be a widespread mechanism among different cell types. Various laboratories have demonstrated that the strength of a 5′ splicing site affects recognition of the upstream intron consistent with a model proposing interactions across the exon [34–36]. Base pairing of the U1 RNA with the 5′ splicing site can stimulate binding of the general splicing factor U2AF^{65} to the polypyrimidine tract of the preceding intron [37]. This inferred interaction between U1 snRNA and U2AF^{65} is indirect and requires additional proteins [37]. In vitro experiments support the possibility that members of the SR family of proteins can provide a bridging function [38,39]. In vivo experiments demonstrating the splicing enhancer effect of purine-rich exonic elements and the importance of the splicing sites have been performed by different groups [3,6,21,40–45]. Dominiski and Kole [43] showed that the presence of either a strong polypyrimidine tract or a consensus 5′ splicing site compensates for the absence of exonic sequences after deletion of the major part of an artificial globin-derived internal exon. In our FN-EDA model the presence of a strong polypyrimidine tract enhanced exon inclusion, although it did not compensate for the absence of the well-defined polypurinic enhancer element. Despite the differences in exon-intron structure, our results are in agreement with those of Dirksen et al. [46,47], who showed by transient transfection experiments that in the case of the bovine growth hormone gene, where an ESE is

located in the fourth and last exon, the purine-rich element compensates for a weak 5′ splicing site in the preceding exon. These contrasts may indicate that particular ESE-splicing site interactions occur in different systems.

In conclusion, the mouse and human FN alternative splicing regulation is essentially conserved. The elements involved are exonic sequences, particularly but not exclusively a polypurinic enhancer, and suboptimal 5′ and 3′ splice junctions. The experiments in vivo indicate a critical role for the cooperation between the EDA polypurinic exonic splicing enhancer and its 5′ splicing site, which predominates over the 3′ splicing site.

These results make approachable the study of the biological significance of alternative splicing using germ line manipulation. Our current knowledge will allow us to radically modify the FN alternative splicing pattern without changing the amino acid sequence of the FN isoforms, and with minimal interference on the RNA sequence.

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